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Quantitation of Hepatitis C Virus in Liver and Peripheral Blood Mononuclear Cells From Patients With Chronic Hepatitis C Virus Infection

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Since the natural history of hepatitis C virus-associated liver disease and the therapeutic responsiveness might vary according to liver and blood mononuclear cells viral levels, it may be important to quantitate viral RNA in liver, blood mononuclear cells and serum, and to compare these data with genotype, biochemical and histologic data. A polymerase chain reaction-based assay available for serum hepatitis C virus RNA quantitation has been optimized to quantitate viral genomes in liver and peripheral blood mononuclear cells from 47 chronic hepatitis C patients. The procedure permitted hepatitis C virus RNA quantitation in freshly isolated mononuclear cells and in total RNA extracted from frozen mononuclear cells and liver tissue. The intrahepatic viral amount (median: 2.6×10^3 copies/μg RNA; range: 0 to 3.6×10^4 copies/μg RNA) correlated significantly with the hepatitis C virus RNA concentration in serum ($r = 0.76$, $P < .001$) but not in mononuclear cells. Viral RNA concentrations in liver ($P < .001$), serum ($P < 0.01$) and PBMC ($P < 0.05$) were significantly higher in hepatitis C virus genotype 1 patients (essentially type 1b) than in non-1 type cases, but were unrelated to biochemical or histologic indexes of disease activity. In conclusion, the optimized assay permit HCV RNA quantitation in liver and peripheral blood mononuclear cells, suggesting that serum viral level is an accurate measurement of intrahepatic viral burden. ***J. Med. Virol. 54:265–270, 1998.***

KEY WORDS: hepatitis C virus (HCV) infection; HCV RNA quantitation; intrahepatic viral load; HCV genotypes; peripheral blood mononuclear cells (PBMC)

INTRODUCTION

The hepatitis C virus (HCV) is a 9.6 kb long single stranded, RNA virus. Diverse HCV genotypes and subtypes have been described [Simmonds, 1995], subtype 1b being the most prevalent in the Iberian peninsula [Pernas et al., 1995]. Chronic HCV infection is a progressive disease resulting in cirrhosis and hepatocellular carcinoma. Response to interferon alpha (IFNα) treatment is approximately 50% in non-cirrhotic patients, with concomitant changes in serum HCV RNA levels [Shindo et al., 1991]. However, most patients relapse after discontinuation of treatment. The natural history of HCV-associated liver disease and the ultimate therapeutic responsiveness might vary according to liver viral burden [Balart et al., 1993], and might depend on whether HCV infects peripheral blood mononuclear cells (PBMC) [Zignego et al., 1992; Castillo et al., 1994; Navas et al., 1994], although this is still controversial [Taliani et al., 1995; Mihm et al., 1996]. Therefore, it may be important to quantitate HCV RNA in liver and PBMC in chronic hepatitis C patients. Different techniques have been used to quantitate HCV RNA in serum and liver, including end-point titration polymerase chain reaction (PCR) [Fong et al., 1991], branched DNA [Idrovo et al., 1996] and, more recently, a dot-blot PCR [McGuinness et al., 1996]. The applicability of a PCR-based assay, currently available for serum HCV RNA quantitation, is described so as to quantitate HCV RNA in paired liver, PBMC and serum specimens from HCV patients. Virus concentrations in liver and PBMC were compared with the corresponding serum viral levels and analysed for HCV genotype, biochemical and histologic data.

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MATERIALS AND METHODS

Patients

A total of 47 untreated, consecutive patients (26 male, 21 female; age range: 23–62 years) with chronic hepatitis C were included in the study. All the patients had anti-HCV (as detected by INNOTEST HCV Ab III™ and confirmed by INNO-LIA HCV Ab III™, Innogenetics NV, Zwijnaarde, Belgium) and serum HCV RNA, as detected by combined reverse transcription and PCR (RT-PCR) techniques as previously described [Navas et al., 1994]. Liver biopsies were obtained from 32 patients by standard procedures. A part of each liver biopsy was submitted for histopathological studies and the rest was frozen immediately in liquid nitrogen until use. Paired serum and fresh PBMC samples were available from 26 patients and the corresponding liver biopsy had been obtained in 11 of these on the day of blood sampling. Only paired serum and liver biopsy were available from the remaining 21 patients. HCV genotyping was undertaken by restriction fragment length polymorphism analysis of RT-PCR products, as described previously [Navas et al., 1997]. This study was conducted in accordance with the Declaration of Helsinki on human experimentation and as approved by the local Ethics Committee.

HCV RNA Quantitation in Fresh and Frozen PBMC Samples

PBMC were isolated from venous blood of patients and healthy donors (negative PBMC control) by gradient centrifugation and washed with phosphate-buffered saline; the last wash was analysed for HCV RNA. HCV RNA was quantitated in duplicate in fresh PBMC samples suspended in 100 μl phosphate-buffered saline, with the AMPLICOR™ HCV MONITOR, according to the supplier's instructions (Roche Diagnostics System, Inc., Basel, Switzerland). The following experiments were performed in order to adapt the AMPLICOR™ HCV MONITOR standard serum protocol: (i) determination of the optimal cell number for the assay, by testing 0.2×10^6, 1×10^6, and 5×10^6 freshly isolated PBMC; (ii) elimination of the possible interference of cellular proteins through an additional extraction step with phenol/chloroform/isoamyl alcohol (25:24:1), which was carried out after the lysis step (in which internal quantitation standard [IQS] is added) and before the addition of isopropanol to the aqueous phase; RNA extraction was carried out in parallel (with and without an additional extraction step) for all the conditions tested in each patient or healthy subject; and (iii) the pellet was resuspended in a smaller volume, from 1 ml to 200 μl, which represents a five-fold increment in the target RNA to be amplified and would consequently increase the assay sensitivity. Taking these modifications into consideration, the formula to calculate the results was:

$$[\text{i}]\ [(\text{absorbance HCV} \times \text{dilution factor})/(\text{absorbance IQS} \times \text{dilution factor})] \times 100 \times 4$$

Samples from 10 patients were used for HCV RNA quantitation in frozen PBMC and its comparison with the results in fresh PBMC. An aliquot of fresh PBMC containing 1×10^7 viable cells was frozen and stored at −80°C until use. PBMC were then thawed and washed with phosphate-buffered saline and total RNA was extracted using the guanidinium thiocyanate method. The amount of total RNA was measured and diluted to a final concentration of 0.75 μg/μl. Quantitation was carried out by adding 2 μl total RNA of each sample to be tested (containing 1.5 μg RNA, which is an amount equal to that used in an "in house" RT-PCR [Navas et al., 1994]) to a mixture containing 197 μl specimen diluent and 1 μl IQS (equal to 25 IQS copies). This 200 μl mixture was then subjected to AMPLICOR™ PCR and subsequent detection. The formula used to calculate the results was:

$$[\text{ii}]\ [(\text{absorbance HCV} \times \text{dilution factor})/(\text{absorbance IQS} \times \text{dilution factor})] \times 25 \times 4$$

and the results were corrected to give quantitation as HCV RNA copies/μg RNA.

HCV RNA Quantitation in Liver Tissue

Liver specimens were cut and homogenized; total RNA was extracted as in the PBMC samples using the guanidinium thiocyanate method. An amount of 1.5 μg total RNA (2 μl) in a 200 μl mixture containing 197 μl specimen diluent and 1 μl IQS was subjected to amplification in duplicate and detected by the AMPLICOR™ HCV MONITOR, as described above. The formula to calculate the results was the same as for frozen PBMC ([ii]) and the results were corrected to give quantitation as HCV RNA copies/μg RNA. The sensitivity of the assay was 7 HCV copies/μg RNA, calculated as the minimum detectable quantity which is 5 standard deviations above the mean copy number of 5 duplicate AMPLICOR™ HCV MONITOR negative controls. The intra- and inter-assay coefficients of variation were similar to those already reported for the standard serum procedure [Colucci, 1996].

Statistical Analysis

Spearman's rank correlation coefficient was used for correlations. The non-parametric Wilcoxon's rank sum test was used to evaluate differences in two independent groups.

RESULTS

HCV RNA Quantitation in Fresh and Frozen PBMC

HCV RNA was quantitated in different cell numbers of freshly isolated PBMC from 6 patients (#1 to #6 in Table I). Without the AES, this procedure showed good correlation in four of the six cases among HCV recovery in 0.2×10^6, 1×10^6, and, to a lesser extent, in 5×10^6

TABLE I. Quantitation Results in Serum, Liver and PBMC Samples, Together with HCV Types, and Biochemical and Histologic Data of the Patients

Patient #	Serum (copies/ml)	Liver biopsy (copies per μg RNA)	PBMC (copies per 1×10^6 fresh cells)	PBMC (copies per (5′NC-μg RNA in RFLP) frozen cells)	HCV type	Histology (Knodell index)	ALT levels (IU/l)
1	3.0×10^5	N.A.[a]	1195	N.A.	1b	N.A.	82
2	2.1×10^4	1592	203	N.A.	1a	CAH[b]	52
3	3.4×10^6	N.A.	885	N.A.	1b	N.A.	174
4	2.1×10^5	N.A.	358	N.A.	1b + 2a	N.A.	54
5	5.5×10^4	4409	1295	N.A.	1a	CAH	114
6	5.6×10^4	N.A.	425	N.A.	1b	N.A.	266
7	1.3×10^5	N.A.	295	N.A.	1a + 1b	N.A.	97
8	4.8×10^4	8	Negative	N.A.	3a	CPH[c] (2)	73
9	2.5×10^5	6468	147	N.A.	1a	CAH (10)	286
10	4.3×10^5	N.A.	29	N.A.	1b	N.A.	343
11	2.4×10^6	7483	86	N.A.	1b	CAH	69
12	5.6×10^4	N.A.	8	N.A.	3a	N.A.	119
13	9.1×10^5	5227	922	N.A.	1b	CPH	51
14	2.7×10^5	6217	829	N.A.	1b	CAH	253
15	3.2×10^5	N.A.	77	N.A.	1b	N.A.	55
16	1.3×10^4	N.A.	31	N.A.	1b	N.A.	89
17	1.0×10^6	N.A.	197	50	1b	N.A.	137
18	2.4×10^5	8563	157	206	1b	CPH	70
19	8.5×10^5	N.A.	63	90	1b	N.A.	115
20	7.4×10^5	N.A.	217	217	1b	N.A.	49
21	1.2×10^6	16606	88	95	1b	CAH (15)	213
22	1.2×10^6	36451	45	89	1b + 3a	CAH (10)	234
23	1.8×10^4	13512	48	54	1b	CAH	84
24	4.2×10^5	N.A.	143	145	1b	N.A.	48
25	4.0×10^5	N.A.	40	28	1b	N.A.	68
26	4.5×10^5	N.A.	54	30	1b	N.A.	95
27	2.7×10^4	1691	N.A.	N.A.	1b	CAH	61
28	2.7×10^4	4157	N.A.	N.A.	1b	CAH	103
29	3.0×10^4	1921	N.A.	N.A.	1b	CPH (3)	49
30	2.5×10^4	1632	N.A.	N.A.	1b	CAH	108
31	3.2×10^4	Negative[d]	N.A.	N.A.	4	CPH (2)	60
32	2.2×10^5	3485	N.A.	N.A.	1b	CAH	89
33	3.0×10^3	18	N.A.	N.A.	4	CAH (6)	107
34	1.4×10^5	2021	N.A.	N.A.	1b	CPH (2)	76
35	1.6×10^4	1642	N.A.	N.A.	1b	CPH	70
36	4.8×10^3	633	N.A.	N.A.	3a	CAH	172
37	3.1×10^3	2962	N.A.	N.A.	1b	CPH (2)	78
38	2.7×10^5	3137	N.A.	N.A.	1b	CAH (11)	398
39	2.1×10^5	2904	N.A.	N.A.	1b	CAH (8)	192
40	8.9×10^3	8	N.A.	N.A.	1a	CAH (13)	92
41	2.3×10^4	2163	N.A.	N.A.	1b	CAH (10)	109
42	1.4×10^5	559	N.A.	N.A.	1b	CAH	236
43	2.2×10^5	6145	N.A.	N.A.	1b	CPH (5)	140
44	4.6×10^5	3629	N.A.	N.A.	1b	CAH (6)	114
45	5.4×10^3	23	N.A.	N.A.	2a	CAH	72
46	1.9×10^5	1207	N.A.	N.A.	1b	CAH	134
47	6.2×10^3	2355	N.A.	N.A.	1b	CAH	303

[a]N.A.: Not available.
[b]CAH: chronic active hepatitis.
[c]CPH: chronic persistent hepatitis.
[d]HCV: RNA detectable by nested-PCR.

cells (data not shown); the assay was negative in the other two cases. In contrast, HCV could be quantitated in all six cases after carrying out the additional extraction step. Thus, the inclusion of this step improved the sensitivity and the reproducibility of the technique, as the HCV RNA concentrations obtained in different cell numbers including the additional extraction step were always higher than those without additional extraction, and the intra-assay coefficients of variation were always lower (data not shown). Accordingly, the assay was subsequently performed in 1×10^6 cells with inclusion of the additional extraction. Finally, fresh cells obtained from patients #7 to #26 in Table I were assayed after the additional extraction, with the pellet being suspended in 200 μl specimen diluent. This modification allowed viral quantitation in PBMC samples with very low HCV amounts (Table I). Overall, the viral load in 26 fresh PBMC samples tested ranged from 0 to 1295 copies/1×10^6 fresh cells (median: 145 copies/1×10^6 fresh cells). No HCV RNA positivity was

found in any of the final PBMC washes, the possibility of contamination with serum-derived viral particles being ruled out.

As shown in Table I, all 10 cases tested were positive in total RNA isolated from frozen PBMC. A statistically significant positive correlation was found between HCV RNA quantitation results obtained in fresh and frozen PBMC (Spearman's correlation coefficient: r = 0.67, $P = 0.033$). However, no significant correlation was found between HCV RNA levels in fresh or frozen PBMC and serum levels (data not shown). HCV RNA concentrations were higher in serum than in fresh or frozen mononuclear cells isolated from an identical amount of blood (1 ml) (ranging from 4.3- to 3820-fold increase, median: 365-fold increase).

HCV RNA Quantitation in Liver Tissue

HCV-specific amplification was obtained in all save one liver biopsy tested (Table I). The negative result obtained was not due to an inefficient extraction (as demonstrated by amplification of cellular β-actin [data not shown]) or to inhibition of the amplification procedure, as the IQS was properly amplified. As shown in Table I, the HCV RNA concentration observed in the liver tissue (excluding the case with a negative result) ranged from 8 to 3.4×10^4 copies/μg RNA (median of 2.6×10^3 copies/μg RNA). As shown in Figure 1A, there was a statistically significant positive correlation between HCV RNA levels in liver and serum samples (r = 0.76, $P < 0.001$). However, no significant correlation was found between HCV RNA levels in fresh PBMC and in liver (data not shown).

According to HCV genotypes (Table I), the liver HCV RNA concentration was significantly higher in HCV type 1 patients (median: 3.1×10^3 copies/μg RNA, range: 8 to 3.6×10^4 copies/μg RNA) than in non-1 type cases (median: 18 copies/μg RNA, range: 0 to 6.3×10^2 copies/μg RNA) (Wilcoxon's rank sum test: $P < 0.01$). HCV 1b accounted for most type 1 cases (Table I); accordingly, HCV 1b patients had significantly higher liver HCV RNA concentration than non-1b cases ($P < .01$) (Fig. 1B), and, in particular, higher than type 3 ($P < .05$), or type 4 ($P < .05$) cases. Similarly, serum HCV RNA concentration was also related to HCV type 1 (median: 2.2×10^5 copies/ml, range: 3.1×10^3 to 3.4×10^6 copies/ml vs. non-1 type: median: 1.9×10^4 copies/ml, range: 3.0×10^3 to 5.6×10^4 copies/ml; $P < 0.01$), and more discernibly in HCV type 1b patients than in the others ($P < 0.01$). Finally, HCV RNA concentration in fresh PBMC was significantly higher in HCV type 1b, and/or 1a patients, than in those with type 3 ($P < 0.05$).

In order to assess whether the amount of HCV RNA in serum, PBMC or liver was related to biochemical or histologic indexes of disease activity, we analysed serum ALT levels and the histologic score. ALT levels did not correlate with HCV RNA in serum, fresh PBMC, or liver samples (data not shown). Similarly, the histologic score did not correlate significantly with HCV RNA levels in liver, fresh PBMC, or serum (data not shown).

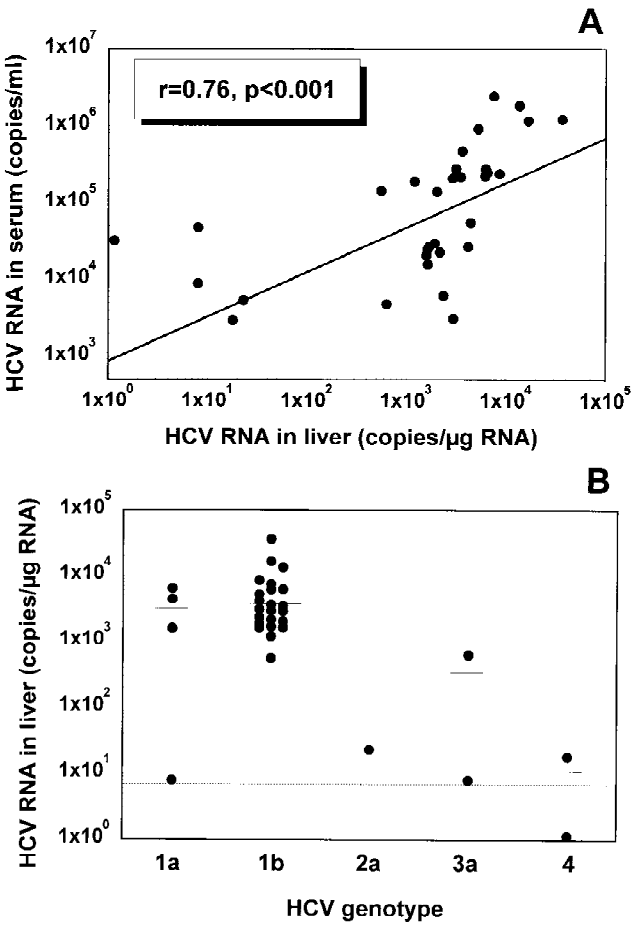

Fig. 1. (A) Correlation between HCV RNA concentrations in serum and liver samples from 32 chronic hepatitis C patients (r, Spearman's correlation coefficient); and (B) Relationship between intrahepatic HCV RNA levels and the infecting genotype. HCV RNA concentration was significantly higher in HCV type 1 patients than in non-1 type cases (Wilcoxon's rank sum test: $P < 0.01$). Horizontal bars represent the median value within each type; dotted line refers to the detection limit of HCV RNA quantitation in liver.

DISCUSSION

A currently available combined RT-PCR assay for quantitation of serum HCV RNA, the AMPLICOR™ HCV MONITOR, was optimized for the quantitation of viral genomes in blood mononuclear cells and liver tissue in patients with chronic hepatitis C. The assay procedure was modified so as to use the appropriate cell number, avoiding interference from the cell matrix, and to reasonably increase the amount of target RNA to be amplified. These modifications reduced the number of cells which must be available for the assay (this is often a limitation) to 1×10^6 fresh cells; at the same time, assay sensitivity was increased, thus preventing HCV undetectability due to a low viral load. This optimized assay has permitted quantitation of HCV RNA in freshly isolated PBMC.

Because fresh PBMC preparations are not always available, and liver biopsies are frozen immediately to preserve RNA integrity, the procedure was adapted to quantitate the viral genomes present in frozen PBMC

and liver tissue and calculated the concentrations on a per μg total RNA basis. The results in frozen PBMC were almost the same as those obtained in fresh PBMC, and these correlated significantly. This fact shows that if PBMC are properly frozen and thawed, no significant degradation of RNA should occur, and frozen PBMC may be used for quantitation of HCV RNA. As PBMC are thought to be an HCV reservoir, the HCV status in PBMC can be evaluated using either fresh or frozen cells. Therefore, monitoring of antiviral effects on PBMC [Castillo et al., 1994] can be carried out with no potential experimental bias due to the use of frozen PBMC samples.

The amount of HCV RNA in liver tissue could be quantitated in all but one biopsy. In that case, the concentration must have been below the detection limit of the technique, as it tested positive by qualitative PCR assay. Over the past few years, several methods have been used to calculate the amount of intrahepatic HCV RNA. These include end-point titration PCR [Fong et al., 1991], branched DNA [Idrovo et al., 1996] and, more recently, a dot-blot PCR [McGuinness et al., 1996]. Diverse methods may give different concentrations of liver HCV RNA, depending on the way the results were calculated and expressed [Fong et al., 1991; Idrovo et al., 1996; McGuinness et al., 1996]. Our results in intrahepatic HCV RNA concentration are comparable to those obtained by branched DNA [Idrovo et al., 1996], but lower than those reported using dot-blot PCR [McGuinness et al., 1996]. Nevertheless, significant correlation was found between HCV RNA concentrations in liver and serum, using the same assay with minor modifications. This finding is in agreement with previous reports [Fong et al., 1991; Idrovo et al., 1996; McGuinness et al., 1996], regardless of the method used. The amount of HCV RNA was higher in serum, or liver, than in PBMC, although the pathogenic relevance of this finding regarding extrahepatic immunologic disorders is still unclear [Muratori et al., 1996].

The amount of HCV RNA in serum and liver was higher in patients with genotype 1, and was more discernible in HCV type 1b than in the rest. Furthermore, the amount of HCV RNA in PBMC was higher in genotype 1 than in genotype 3 patients. HCV genotype 1 may have biological characteristics which result in a higher HCV replication. This fact is of interest, as therapeutic response probably depends on the infecting genotype, the virus load, or a combination of both factors [Simmonds, 1995], although it cannot be ruled-out completely that the assay can actually quantitate different genomes with unequal efficiency. In this sense, Hawkins et al. [1997] have compared recently different methods for the quantitation of HCV in plasma among individuals infected with HCV genotypes 1, 2 and 3. They concluded that the AMPLICOR™ HCV MONITOR assay did not quantify efficiently genotypes 2 and 3, compared with genotype 1. Based on their results, these authors have proposed correction factors that might be applied to data obtained in clinical specimens: multiplying virus load by a factor of 9 for type 2 samples, and by a factor of 12 for type 3 samples. Considering this correction, we did not observe differences among genotypes in serum HCV RNA concentrations, in agreement with recent reports using branched DNA [Detmer et al., 1996; Smith et al., 1996; Hawkins et al., 1997]. However, the liver HCV RNA concentration remains significantly higher in patients with genotype 1, even after correction for values of samples with types 2 and 3. Our findings suggest that true biological differences between genotypes might occur at the site of infection by HCV, but this important issue deserves further investigation.

Finally, the concentrations of HCV RNA in liver, serum and PBMC did not correlate with biochemical (ALT values) or histologic indices of disease activity. This is in agreement with the hypothesis that host immune responses to HCV, but not HCV itself, are more relevant to pathogenicity. The data of this study confirm that the amount of viral genome can be reliably quantitated in liver tissue and blood mononuclear cells and that serum viral levels are an accurate measurement of intrahepatic viral levels during chronic HCV infection.

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